Sex Differences in Serotonin (5-HT) Activity During Safety Learning

Author: Kayla Dana Fernando

Sex Differences in Serotonin (5-HT) Activity During Safety Learning

Kayla Dana Fernando

An undergraduate thesis

submitted to the Faculty of

the Department of Biology

in partial fulfillment

of the requirements for the degree of

Bachelor of Science in Biology

Boston College
Morrissey College of Arts and Sciences

May 2018

Sex Differences in Serotonin (5-HT) Activity During Safety Learning

Kayla Dana Fernando

Advisor: John P. Christianson, Ph.D.

Patients with posttraumatic stress disorder (PTSD) often show impaired ability to discriminate between "danger" and "safety" cues. Women are more than twice as likely to be diagnosed with PTSD as compared to men; however, translational research has largely relied on the use of male subjects despite evidence of sex differences in fear-motivated behaviors. Serotonergic activity, originating in the dorsal raphe nucleus (DRN) of the central nervous system (CNS), has been found to modulate fear discrimination in males and may contribute to sex differences observed in a Pavlovian fear discrimination paradigm. In this study, male and intact female Sprague-Dawley rats were exposed to fear conditioning with (CS+/CS-) or without (CS+) a safe conditioned stimulus, then subsequently sacrificed for immunohistochemical analysis of serotonergic activity via quantification of tryptophan hydroxylase (TPH) and Fos in the DRN. Females exhibited more rapid and robust discrimination between the CS+ danger cue and CS- safety cue as compared to males. Regardless of condition, females had more double-labeled TPH+Fos cells compared to males, but males had larger variation in TPH+Fos expression compared to females. A parabolic function for TPH+Fos counts predicted fear discrimination in males, but not females, reinforcing the view that serotonin is a modulator of safety-related behavior in males.

TABLE OF CONTENTS

LIST OF FIGURES

ACKOWLEDGEMENTS

I would like to thank my thesis advisors, John P. Christianson, Ph.D., and Allison R. Foilb, for their guidance throughout this project. I would also like to thank the Boston College Undergraduate Research Fellowship for financial support, the staff of the Boston College Animal Care Facility for their animal care, and Bret Judson for his consultation on cell imaging and image analysis. In addition, I would like to acknowledge the other individuals who I came to know during my time in the lab, for their friendship, inspiration, and support as I prepared my own thesis.

To all, thank you for your endless (im)patience with me throughout this journey to help me become not only a great scientist, but a good one as well.

1.0 INTRODUCTION

Posttraumatic stress disorder (PTSD) is a mental health disorder that an individual may develop after witnessing or experiencing an extremely stressful or traumatic event such as combat, sexual assault, or vehicular accidents. One distinguishing characteristic of PTSD is the inability to distinguish between danger and safety (Jovanovic et al., 2012)—people with PTSD often experience the "fight-or-flight" stress response even when in a safe environment as a result of a "trigger" in the surroundings that reminds him or her of the traumatic event. Research aimed at identifying the biological mechanisms by which stressors reduce the efficacy of safety signals may lead to new targets for therapy of PTSD.

Associative safety learning, based on Pavlovian conditioning (Pavlov, 1927), is often used as an experimental model for studying the impaired fear behaviors endemic to PTSD and the neural mechanisms that underlie them. Fear discrimination is one instance, in which a subject is presented with an emotionally neutral conditioned stimulus (CS) (e.g., a flashing LED light) paired with a salient unconditioned stimulus (US) (e.g., a footshock) such that the subject learns to associate the "danger" cue with impending aversive stimulation—this becomes the CS+. A "safety" cue (e.g., a unique tone) that is not paired with an aversive US, and so does not predict aversive stimulation, becomes the CS-. Neurotypical individuals readily distinguish between the CS+ and CS-, but those with PTSD are unable to exhibit the same level of fear discrimination. Experimental animals are subjected to this fear discrimination paradigm to further study PTSD.

The basolateral amygdala's (BLA) evolutionary function and the modulatory effects of serotonin (5-hydroxytryptamine; 5-HT) have been identified as key components in the regulation of fear behaviors (Lowry et al., 2005; Homberg, 2012; Bocchio et al., 2016), with the continued use but controversial efficacy of selective serotonin reuptake inhibitors (SSRIs) to treat anxiety and depressive-like symptoms (Möller, 2009) indicative of the fact that we have yet to have a firm understanding of the neurocircuitry that underlies such behaviors.

Additionally, results from translational research have come from the almost exclusive use of male subjects, even though women are more than twice as likely as men to be diagnosed with PTSD due to various physiological and psychosocial factors (Breslau et al., 1999b; Kessler et al., 2012) and other studies have replicated the phenomenon that females exhibit more robust fear discrimination as compared to males (Day et al., 2016; Foilb et al., 2017).

In this study, we examined potential sex differences in the dorsal raphe nucleus (DRN), the origin of 5-HT in the central nervous system (CNS), that could explain observed sex differences in a fear discrimination paradigm.

1.1 SEROTONIN (5-HT)

5-HT is a monoamine neurotransmitter synthesized from tryptophan by tryptophan hydroxylase 2 (TPH), a CNS-specific TPH isoform (Walther and Bader, 2003). During synaptic transmission, 5-HT is released from the presynaptic neuron and binds to its associated receptors on the postsynaptic membrane. 5-HT receptor subtypes

have been classified based on their coding sequences, protein morphologies, regional distributions, pharmacological profiles, and modulatory effects on brain functions that give rise to animal behaviors (for reviews see Boess and Martin, 1993; Barnes and Sharp, 1999). It is argued that the specificity of signaling between 5-HT and its receptors mainly depends on general mechanisms of synaptic transmission—namely, the rate of 5-HT synthesis and reuptake, extracellular concentration of the neurotransmitter, diversity and distribution of its receptors, and specific methods of transmission throughout the CNS.

5-HT can be transmitted by "wired transmission," characterized by transmission at synapses, and "volume transmission," characterized by transmission through the extracellular space that includes non-synaptic receptors (Agnati et al., 1995; 2010). 5-HT released from junctional (i.e., synaptic) and non-junctional sites has been shown to escape the synaptic cleft and diffuse into the extracellular space in a paracrine fashion until reuptaken into the presynaptic neuron by ATP-dependent 5-HT transporters. These two modes of transmission may represent complementary modes of communication, with the former involved in short-term signaling and the latter involved in long-term modulation of cellular networks (Fuxe et al., 2007).

Serotonergic neurons originating in the pons of the brainstem—an area containing the DRN—project to the forebrain. A rostral and ventral pathway emerge from this group of neurons, with a longitudinal rostral projection running through the medial forebrain to innervate the BLA (Charnay and Léger, 2010), which contains a dense meshwork of serotonergic axons and 5-HT transporters (Smith and Porrino, 2008). Neurons from the DRN that project to the BLA originate from the dorsomedial part of the DRN (Abrams et

al., 2004), are neurochemically distinct (Commons et al., 2003), and receive unique afferent input (Peyron et al., 1997).

1.2 NEUROBIOLOGY OF FEAR CONDITIONING IN THE BLA

The BLA receives primarily cortical, hippocampal, and sensory inputs, with most of its outputs projecting to the brainstem and brainstem-driving intermediate structures (Swanson and Petrovich, 1998). The ability for the BLA to assign valence to sensory stimuli—particularly those associated with emotionally charged events—involves crosstalk with other brain structures and modulation by neurotransmitters such as 5-HT, making it an area of interest in safety learning (LeDoux, 2007).

Immunohistochemical and electrophysiological techniques have distinguished between two main types of neurons within the BLA. Pyramidal neurons in the BLA, which comprise approximately 70% of the structure's cell population, are immunoreactive to glutamate, suggesting that they are excitatory in nature (Smith and Paré, 1994). Conversely, local interneurons in the BLA are immunoreactive to γ-aminobutyric acid (GABA) (McDonald and Augustine, 1993), suggesting that they are inhibitory in nature.

Plastic events within the BLA are thought to be necessary in enabling the CS to become associated with the nociceptive and emotional valence of the US and trigger the behavioral, autonomic, and endocrine characteristics of the stress response. Through exposure to stressful stimuli, the BLA can promote the establishment of long-term fear memory in a process known as consolidation. Transmission of the sensory information of

the CS to the BLA leads to the release of glutamate from sensory inputs onto AMPA receptors. If a strong enough US is presented to the neuron at the same time as the CS, an increase in excitatory input causes the removal of the Mg^{2+} block from nearby NMDA receptors and allows extracellular Ca^{2+} to enter the postsynaptic membrane through the NMDA receptor and other Ca^{2+} ion channels. Increased concentrations of intracellular Ca^{2+} result in phosphorylation cascades which have further downstream effects within the cell via CaMKII activity for an overall enhancement of synaptic transmission (Manilow et al., 1989, Lisman et al., 2002). This process, known as long-term potentiation, induces the synthesis of proteins that play a role in synaptic plasticity and memory consolidation (Stanton and Sarvey, 1984), such as new AMPA receptors which are inserted into the postsynaptic membrane to facilitate subsequent Ca^{2+} influxes (Shi et al., 1999, Sanderson et al., 2016) in a positive feedback loop.

McKernan and Shinnick-Gallagher (1997) found that rats that had undergone fear conditioning showed enhanced amplitude of synaptic currents in amygdaloid neurons *in vitro*, and Maren (1999) and other groups later found that administration of NMDA receptor antagonists into the BLA effectively prevented fear conditioning. Activity in local inhibitory circuits has also been found to mediate fear behaviors, as different treatments that increased GABA transmission were found to impair the acquisition (Sanger and Joly, 1985) and expression (Harris and Westbrook, 1999; 2001) of conditioned fear.

The BLA has also been shown to exhibit synaptic plasticity after exposure to safety cues. Ostroff et al. (2010) identified morphological changes in the dendritic spines of excitatory amygdaloid neurons, where fear conditioning resulted in larger spines but

presentation of unpaired cues resulted in smaller spines. On a macroscopic scale, Sangha et al. (2013) identified unique populations of neurons whose firing rates encoded the danger cue, the safety cue, or both. As the spatiotemporal properties of long-term potentiation have been shown during CS-US association in fear conditioning (Rogan et al., 1997; Shin et al., 2006), this mechanism can also serve as a working model for neural activity during fear discrimination and fear memory consolidation in the BLA.

1.3 SEROTONERGIC MODULATION OF FEAR BEHAVIORS

Using retrograde tracers injected into the anterior part of the BLA, Abrams et al. (2005) identified a "shell" subregion of the mid-DRN as the potential origin of serotonergic modulation of fear behaviors, providing further anatomical support to the existing hypothesis that 5-HT plays a modulatory role in a range of emotionally salient behavioral responses. Other studies in which direct application of 5-HT or related drugs into the BLA influenced the stress response provide pharmacological support for serotonergic modulation of fear behaviors.

The DRN-BLA system is affected by exogenous stimuli and drugs in a time- and dose-dependent manner. Electrical stimulation of the DRN inhibited neural activity in the BLA *in vivo* (Wang and Aghajanian, 1977), an effect replicated with late acute administration of 5-HT into the BLA that increased depolarization in interneurons and hyperpolarization in pyramidal neurons (Rainnie, 1999). Axons that innervate pyramidal neurons and interneurons in the BLA were immunoreactive to 5-HT, and serial sectioning transmission electron microscopy identified clusters of synaptic vesicles containing the

neurotransmitter (Muller et al., 2007). Therefore, if an increase in 5-HT were to inhibit the BLA, then synaptic plasticity would be impaired during learning, which would in turn impair short-term fear discrimination long-term fear memory consolidation.

Exposure to stress, which produces long-lasting behavioral changes in experimental animals, has also been shown to involve the DRN (Maier and Watkins, 2005) and serotonergic modulation in the BLA (Kawahara et al., 1993; Amat et al., 1998; Zanoveli et al., 2009). Other pharmacological manipulations revealed that 5-HT levels in the BLA after inescapable stress were mediated by 5-HT_{2C} receptors (Campbell and Merchant, 2003; Christianson et al., 2010), which could improve performance in safety learning (Foilb and Christianson, 2015). Acute administration of SSRIs which transiently increased 5-HT levels in the BLA also increased anxiety-like behavior (Burghardt et al., 2004; 2007), and Vicente and Zangrossi (2012) connected this effect of SSRIs with activity of 5-HT_{2C} receptors in the BLA. These results suggest that transitory increases in 5-HT in the BLA facilitate both the acquisition and expression of fear.

Stress may cause an imbalance in the DRN-BLA system by downregulating local inhibitory 5-HT_{1A} autoreceptor activity (Rozeske et al., 2011; Vicente and Zangrossi, 2014; Li et al., 2012; Li et al., 2016) in the DRN to increase the firing rate of serotonergic neurons. The resulting increase of 5-HT that is released may lead to hyperexcitation of the DRN-BLA system and interfere with other properties of synaptic plasticity such as firing rate, gene expression, phosphorylation cascades, protein scaffolds, and lipid rafts to influence the establishment of long-term potentiation during fear discrimination (for review see Lesch and Waider, 2012).

A dual activity-increasing effect of serotonergic transmission of different neuron types in the BLA was indirectly suggested by Hale et al. (2010), who found that administration of anxiogenic drugs induced correlated increases in the expression of c-Fos (hereafter referred to as "Fos"), a protein biomarker of neural activity expressed by the immediate early gene of the same name, in BLA interneurons. Fos was also strongly induced in BLA pyramidal neurons which have been shown to be activated by acute and repeated restraint stress (Reznikov et al., 2008). In conjunction with Abrams et al.'s (2005) similar observations in the DRN, these results provide further evidence of crosstalk within the DRN-BLA system. Hale et al. (2010) suggested that Fos expression in glutamatergic pyramidal neurons might be involved in the stress response itself, while the activation of GABAergic interneurons could contribute to its termination, corroborating the model of neuronal interaction in the BLA proposed by Rainnie (1999).

Considering the synthesis of 5-HT, sex differences in the serotonergic system may also be explained by TPH expression. Female *TphII*$^{-/-}$ mice subjected to chronic mild stress exhibited increased anxiety-like behavior, reactivity to the US during conditioning, and levels of corticosterone metabolites as compared to males, suggesting that activation of the hypothalamic-pituitary-adrenal axis by 5-HT deficiency is more pronounced in females (Gutknecht et al., 2015). This hypothesis was supported by an observable decrease in Fos in the DRN in females after injections of corticotropin-releasing factor (Howerton et al., 2014). These results suggest that other hormones may also influence BLA activity and account for sex differences in the stress response.

1.4 AIM OF CURRENT STUDY

While there is extensive work on the role of serotonergic modulation in the BLA during fear discrimination, most of these studies fail to consider the possibility of sex differences in this brain region. Additionally, no studies have examined serotonergic activity in the DRN as a neural correlate for observed sex differences in fear discrimination. Therefore, identifying sex differences in serotonergic activity at the likely origin of serotonergic modulation of fear behaviors would lead to a better understanding of the neurocircuitry of fear discrimination and the manifestation of PTSD in the human population.

If pharmacological studies have shown that administration of a 5-HT_{2C} receptor antagonist in fear-related structures such as the BLA could improve male subjects' ability to discriminate (Foilb and Christianson, 2015), we hypothesized that females may already have low levels of 5-HT in the BLA that could explain their robust fear discrimination.

In order to study sex differences in serotonergic activity, we first replicated sex differences in a fear discrimination paradigm. To allow for serotonergic modulation in the DRN-BLA system to function in its endogenous state, there was no experimental manipulation of the animals beyond that necessary to conduct behavioral testing. By performing immunohistochemistry on fixed DRN sections of all experimental animals, we could confidently identify serotonergic cells in the region that had been activated as a direct result of fear discrimination. By comparing average serotonergic activity between the sexes given the same conditioning treatment and correlating the number of activated serotonergic cells to the animal's ability to discriminate, we could see if serotonergic activity in the DRN varied between the sexes and if it was an accurate predictor of fear

discrimination. Additionally, differences in morphology, TPH expression, and global activity in the DRN could also explain discrepancies in fear discrimination.

2.0 METHODS & MATERIALS

2.1 SUBJECTS

Intact male and normally cycling female adult Sprague-Dawley rats (*n* = 24/sex, Taconic Biosciences, Germantown, NY) were received at approximately 7 weeks old, kept in the same vivarium, and allowed 7-10 days to acclimate to the new environment before any experimental procedures. Animals were housed in isosexual pairs in plastic tub cages under a 12:12 light/dark cycle. Food and water were provided *ad libitum*. All experimental procedures were approved by the Boston College Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 APPARATUS

Fear discrimination conditioning occurred in 10 x 11 x 6 in. (L x W x H) black plastic chambers with wire mesh lids and a floor of stainless steel bars attached to a shocking grid (Model H10-11R-TC-SF, Coulbourn Instruments, Whitehall, PA) surrounded by a 15 x 12 x 27 in. (L x W x H) enclosure with ventilation and stochastic

noise (~55 dB) provided by a small fan. Each enclosure was equipped with two infrared LED lights (CMVision Model IR30) and one overhead camera (Model VX-5000, Microsoft, Redmond, VA) modified with an infrared passing filter to detect infrared light. A white LED light array (Model LPL620WTHD) and speakers affixed to the top of each enclosure provided conditioning stimuli.

2.3 CONDITIONING

The animals were assigned to the following treatments (n = 8/sex): fear discrimination conditioning consisting of 15 shock-paired CSs and 15 unpaired CSs (CS+/CS-), fear conditioning consisting of 15 shock-paired CSs (CS+ only), or a control group which was exposed to the fear discrimination stimuli without any shock (control). The conditioning stimuli were either auditory (10-ms white noise pips, 2 Hz, 75 dB) or visual (20-ms on/off flashing LED light, 264.0 Lux) cues which were counterbalanced across treatments. Freezing was video recorded and scored using ANY-Maze software (Version 4.99, Stoelting, Wood Dale, IL). All conditioning sessions were performed in the morning.

Fear discrimination conditioning consisted of 15 presentations each of the CS+ and CS-. Trials began with a 5-second 1 kHz (75 dB) tone, followed by a 15-second cue presentation of either the CS+, which always co-terminated with a 500-ms, 1.2 mA scrambled footshock, or the CS-, which was never paired with a footshock. Animals were presented with the auditory and visual cues in quasi-random order such that no cue was presented more than twice in series, and a 70-second inter-trial interval separated each

cue presentation such that the session lasted 45 minutes. Fear conditioning trials began with the 5-second tone, followed by presentation of the CS+ for 15 seconds. Each CS+ presentation co-terminated with a footshock. An extended inter-trial interval of randomized length was presented after each cue presentation such that the full session lasted 45 minutes. The control treatment was exactly as the fear discrimination conditioning without any footshocks.

2.4 ESTROUS CYCLE DETERMINATION AND ELISA TESTING

Vaginal smears were obtained to determine estrous phase in the female rats. Estrous samples were collected at approximately the same time each day, 1 hour after the conditioning session. An eye dropper with 0.5 mL of saline solution was inserted approximately 5 mm into the vagina. Vaginal fluid was pipetted 3-4 times, placed on microscope slides, and coverslipped. Vaginal cytology was observed using light microscopy (American Optical Corporation, Model 1051, Buffalo, NY) at 10x magnification to determine estrous phase in accordance with guidelines described by Marcondes et al. (2002).

After performing the vaginal smear, rats were anesthetized with tribromoethanol (i.p.), the thoracic cavity was opened, and approximately 2 mL of blood were collected via cardiac puncture. Blood was allowed to clot at room temperature and then centrifuged at 10,000 x g for 10 minutes at 4°C. Serum supernatant was preserved for later ELISA testing at -20°C (data not included).

2.5 IMMUNOHISTOCHEMISTRY

After blood collection, rats were transcardially perfused with approximately 200 mL of ice-cold PBS followed by 4% paraformaldehyde solution. Brains were extracted, post-fixed overnight and transferred to 30% sucrose solution. Brains were flash-frozen in 2-methylbutane, and 40 µm slices (6.12 mm Bregma – -8.76 mm Bregma, Paxinos and Watson, 2006) were collected from a freezing cryostat (-20ºC, Leica Biosystems, Wetzlar, Germany). 1 out of 7 slices was directly mounted onto a gelatin subbed microscope slide for further cresyl violet staining and coverslipping with Permount (Thermo Fisher Scientific) for reference.

Immunohistochemistry was performed at room temperature on DRN (B-6.84 mm – B-8.52 mm) slices. Sections were washed in 0.1 M PBS containing 0.1% Triton-X 100 (PBST) and blocked in 5% normal donkey serum (Jackson ImmunoResearch) for 1 hour. Sections were then incubated in sheep anti-TPH primary antibody (1:1000; Sigma-Aldrich; Product No. T8575-1VL; Lot No. SLBP0011V) overnight at 4ºC. Sections were washed in PBST, then incubated in donkey anti-sheep secondary antibody (1:200; Jackson ImmunoResearch; AlexaFluor® 488 AffiniPure Donkey Anti-Sheep IgG (H+L); Product No. 713-545-147; Lot No. 133387) for 2 hours in the dark. After another PBST wash, the sections were blocked in 5% normal goat serum (Jackson ImmunoResearch) for 1 hour. Sections were then incubated in rabbit anti-c-Fos primary antibody (1:5000; Millipore; Product No. ABE457; Lot No. 2905394) overnight at 4ºC. Sections were washed in PBST, then incubated in goat anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch; AlexaFluor® 594 AffiniPure Goat Anti-Rabbit IgG (H+L); Product

No. 111-585-045; Lot No. 121581) for 2 hours in the dark. Sections were floated onto unsubbed slides and coverslipped with Vectashield® mounting medium with DAPI.

2.6 CELL IMAGING

Epiflourescent DRN image stacks were acquired with a Plan-Apochromat 10x/0.45 NA objective on a Zeiss AxioImager Z2 microscope (Thornwood, NY) equipped with a Hamamatsu ORCA-R2 CCD camera (Bridgewater, NJ). An Apotome was used for optical sectioning. Presence of Fos, TPH, and double-labeling of TPH and Fos was measured with ImageJ software (National Institutes of Health). To calculate Fos density, a Fos:area ratio was obtained by dividing the number of labeled Fos nuclei by the DRN area (mm^2) and used as a measure of global activity. Serotonergic activity was measured by quantifying the number of double-labeled TPH+Fos cells, dividing this number by the total number of TPH cells, and converting this ratio into a percent. To calculate TPH density, a TPH:area ratio was obtained by dividing the number of labeled TPH cells by the DRN area (mm^2).

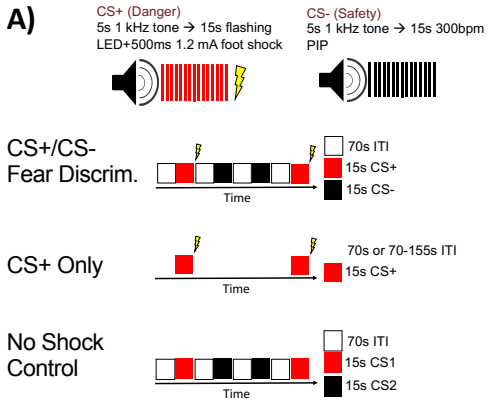
2.7 STATISTICS

Statistical analyses were performed using GraphPad Prism 7. By-trial freezing data were analyzed by three-way analysis of variance (ANOVA) with sex as a between-subjects variable and cue type and trial as within-subjects variables. Two-way ANOVAs

were used to analyze freezing across conditioning sessions, Fos densities, percent TPH+Fos, DRN areas, and TPH densities, with sex as a between-subjects variable and cue as a within-subjects variable. Significant main effects and interactions ($p < 0.05$) were followed by Sidak or Tukey post-hoc comparisons. Unpaired t-tests were used to analyze DRN areas and TPH densities, and *F*-tests were used to compare variances. Linear and nonlinear relationships between fear discrimination and percent TPH+Fos were evident in the data. These were assessed with Pearson's r and a nonlinear regression fit of a quadratic function; goodness of fit was quantified with R-squared.

3.0 RESULTS

3.1 FEMALES EXHIBIT MORE RAPID AND ROBUST FEAR DISCRIMINATION COMPARED TO MALES



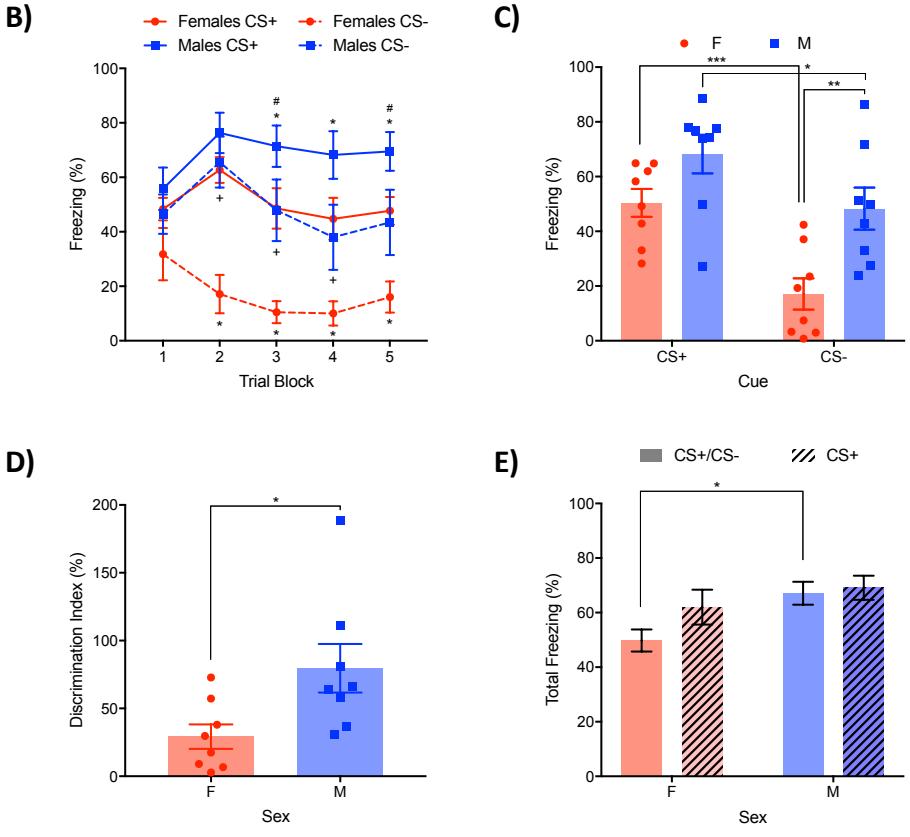


Figure 1. Differences in fear discrimination. A) Experimental designs of the three different conditions (n = 8/sex). **B)** Freezing averages (±SEM) to the CS+ and CS- averaged across trial blocks (3, 15-s cue presentations) for animals given CS+/CS- conditioning. Females discriminated between CS+ and CS- by trial block 2 of each cue (*ps < 0.006), while males did not make this discrimination until trial block 3 (*ps < 0.04). Males displayed significantly more freezing compared to females to both the CS+ ($^{\#}p$s < 0.05) and CS- (^{+}ps < 0.05). *significant discrimination within sex, $^{\#}$significant sex difference on CS+, $^{+}$significant sex difference on CS-. **C)** Mean and individual replicates of freezing to CS+ and CS- during conditioning. Freezing to each cue was averaged over 15 presentations and converted to a percentage of time (±SEM). Females exhibited greater discrimination between CS+ and CS- (***p < 0.001) compared to males (*p < 0.05). Males exhibited greater freezing to the CS- compared to females (**p < 0.01). **D)** Mean and individual replicates of percent discrimination indices ($\frac{\text{CS}-\text{freezing}}{\text{CS}+\text{freezing}} \times 100\%$) (±SEM) during conditioning. An index below 100 signifies reduced freezing to the CS- compared to the CS+, and therefore successful fear discrimination. Females showed more robust discrimination compared to males (*p < 0.05). **E)** Comparison of percent freezing (±SEM) over conditioning sessions between CS+/CS- and CS+ animals. CS+/CS- males exhibited similar levels of fear compared to CS+ males, but CS+/CS- females exhibited less total fear compared to CS+ females. A sex difference in freezing in CS+/CS- animals was indicative of differences in fear discrimination (*p < 0.05).

Experimental designs of the CS+/CS-, CS+, and no shock conditions are described in Figure 1A. No animals were excluded from behavioral analysis. Estrous phase was not found to have a significant effect on fear discrimination in females (data not shown). For by-trial data in the CS+/CS- condition (Figure 1B), time spent freezing was analyzed in 5 trial blocks, each containing 3, 15s cue presentations. Data were aggregated and sorted by cue presentation, and freezing was determined by comparing freezing levels to the CS+ compared to the CS-. A significant difference in freezing between the two cues indicated the presence of fear discrimination. There was a main effect of cue ($F_{(1,14)}$ = 28.979, p < 0.001), sex ($F_{(1,14)}$ = 10.137, p =0.007), trial ($F_{(4,56)}$ = 6.485, p < 0.001), and a trial by sex interaction ($F_{(4.56)}$ = 3.254, p = 0.018). Discrimination was significant in females from trial blocks 2-5 (T2 p = 0.001; T3 p = 0.002; T4 p = 0.002; T5 p = 0.006), whereas males did not show discrimination until trial

blocks 3-5 and were not as robust as females (T3 $p = 0.038$; T4 $p = 0.005$; T5 $p = 0.020$). Males displayed higher freezing compared to females to both the CS+ (T3 $p = 0.049$; T5 $p = 0.026$) and CS- (T2 $p = 0.001$; T3 $p = 0.008$; T4 $p = 0.046$).

Average time spent freezing to either the CS+ or the CS- was calculated by converting the average freezing score over the 15s cue presentation to a percentage. Females exhibited a more significant difference in freezing between the CS+ and CS- (Figure 1C) ($p = 0.0006$) compared to males ($p = 0.0254$), with males exhibiting more freezing to the CS- compared to females ($p = 0.0040$).

For the CS+/CS- condition, a discrimination index was calculated by dividing the amount of time freezing to the CS- over the amount of time spent freezing to the CS+ over the 45-minute session, then converting this ratio into a percent. A lower index indicates more robust fear discrimination, while a higher index indicates the opposite. Females had a lower discrimination index as compared to males (Figure 1D) ($p = 0.0245$), freezing less to the CS- compared to the CS+ and confirming that females show robust fear discrimination.

To determine if freezing during CS+/CS- conditioning differed from that in CS+ only conditioning, we compared overall time spent freezing (percent of total test time) (Figure 1E). Freezing was similar between groups in males, but females exhibited less total fear in the CS+/CS- condition than the CS+ only condition, a reflection of the greater fear inhibition to the CS- evident in Figure 1C. There is a significant sex difference within the CS+/CS- group, with females spending less total time freezing compared to males ($p = 0.0355$) further supporting previous results of more robust fear discrimination.

3.2 MALES AND FEMALES HAVE DIFFERENT CORRELATIONS BETWEEN CONDITION AND DRN ACTIVITY

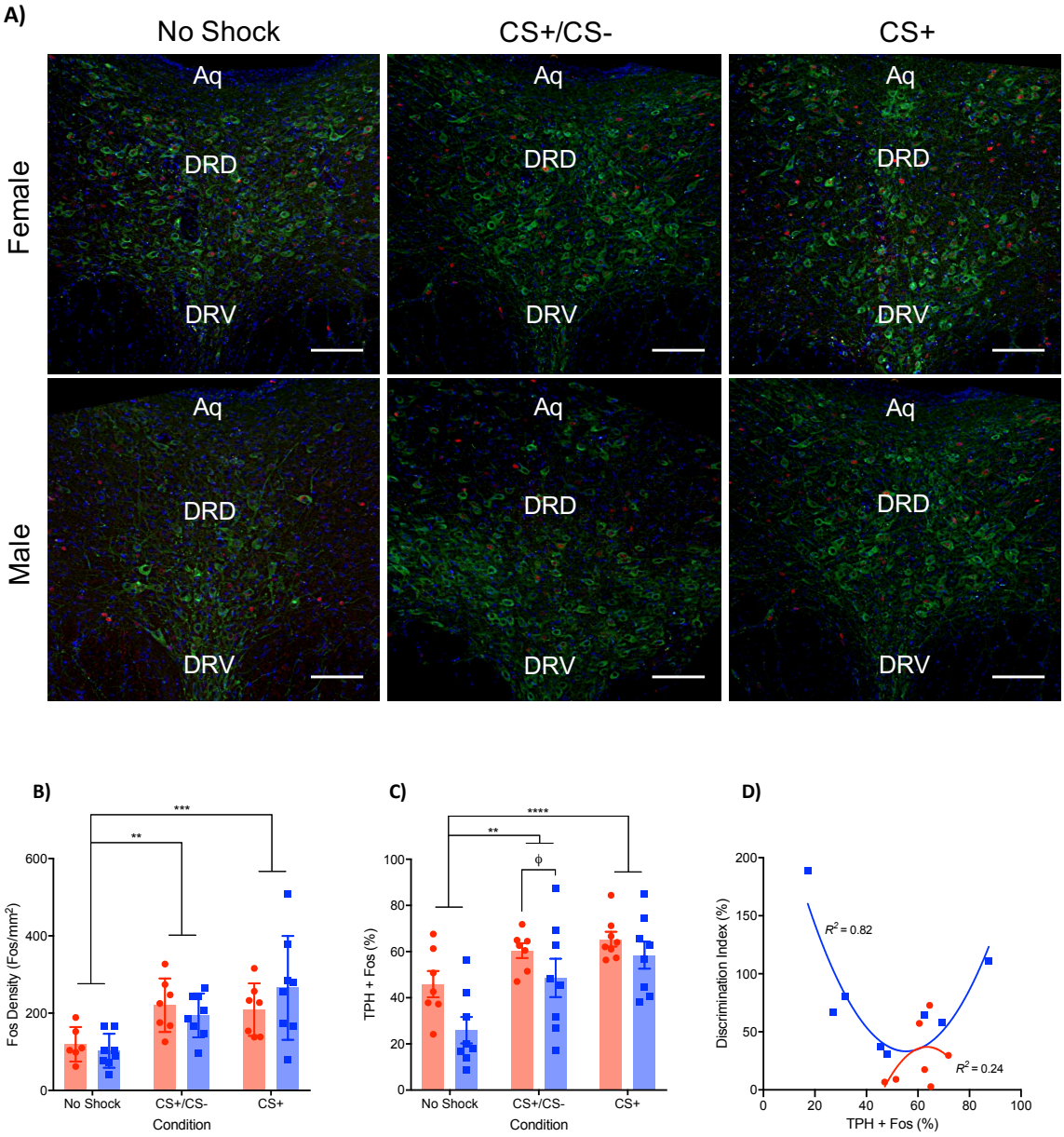


Figure 2. Correlation between condition and DRN activity. A) Representative epifluorescent stack images of DRN sections (approx. -8.00 mm Bregma) from male and female rats under various conditions at 10x magnification. Serotonergic activity was measured by quantifying double-labeled TPH+Fos (green+red) cells with a DAPI (blue) counterstain. Aq: aqueduct; DRD: dorsal dorsal raphe; DRV: ventral dorsal raphe. Scale bar: 100 μm. **B)** Comparison of average Fos densities (Fos/mm^2) (±SEM) between sexes and across conditions. There were no sex differences in overall Fos immunoreactivity within each condition, but there were significant changes due to condition (**$p < 0.01$, ***$p < 0.001$). **C)** Comparison of percent TPH+Fos (±SEM) between sexes and across conditions. There were no sex differences in overall TPH+Fos immunoreactivity within each condition, but there were significant changes due to condition (**$p < 0.01$, ****$p < 0.0001$). Notably, CS+/CS- males had larger variation compared to CS+/CS- females ($p < 0.05$). **D)** This variation was furthered explored by correlating percent TPH+Fos to discrimination index. Males, but not females, exhibited a parabolic correlation between serotonergic activity and discrimination index as indicated by R-squared values.

One female from the "no shock" condition and one female from the CS+/CS- condition were excluded from immunohistochemical analysis due to high background fluorescence. Immunohistochemistry in DRN revealed successful cytoplasmic labeling of TPH and nuclear labeling of Fos (Figure 2A). Two-way ANOVAs of Fos densities (Fos/mm^2) (Figure 2B) and percent TPH+Fos (Figure 2C) revealed that there were no sex differences in either global or serotonergic activity within each condition, respectively; however, there were significant changes due to condition. There was a significant increase in Fos density with more exposure to the CS+ (Figure 2B, CS+/CS- to no shock, $p = 0.0051$; CS+ to no shock, $p = 0.0002$), a trend that was reflected in percent TPH+Fos (Figure 2C, CS+/CS- to no shock, $p = 0.0062$; CS+ to no shock, $p < 0.0001$).

Interestingly, an *F*-test revealed that CS+/CS- males had larger variation in percent TPH+Fos as compared to CS+/CS- females (Figure 2C) ($p = 0.0237$). We further explored this condition by correlating an animal's percent TPH+Fos to its discrimination index as previously calculated (Figure 2D). A second-order nonlinear quadratic

correlation was determined to be the best fit for males accounting for a majority of data variability ($R^2 = 0.82$), as opposed to our original hypothesis of a linear correlation (data not shown). A high R-squared value for males, but not females ($R^2 = 0.24$), is suggestive of a parabolic correlation between percent TPH+Fos and fear discrimination.

3.3 DRN MORPHOLOGY CANNOT ACCOUNT FOR DIFFERENCES IN SEROTONERGIC ACTIVITY

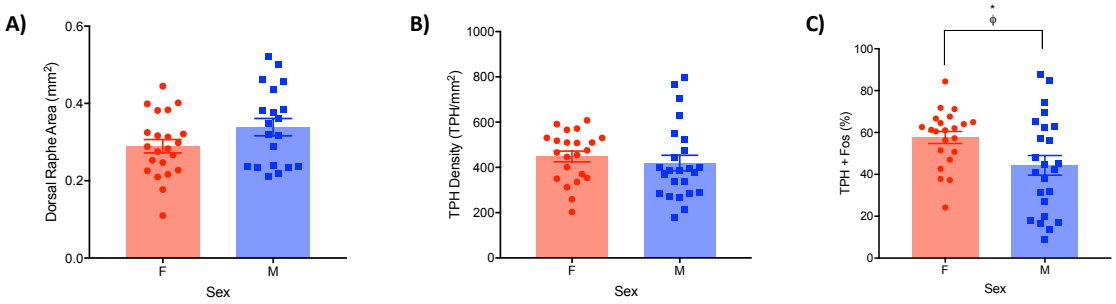


Figure 3. DRN anatomy. A) Average DRN areas (mm^2) (±SEM), regardless of condition. Males and females had similar DRN areas. **B)** TPH densities within the DRN (TPH/mm^2) (±SEM), regardless of condition. Males and females had similar TPH densities. **C)** Percent TPH+Fos (±SEM), regardless of condition. Females had higher TPH+Fos immunoreactivity compared to males, and therefore higher serotonergic activity (*$p < 0.05$). Males had larger variation in serotonergic activity compared to females ($\phi p < 0.05$).

We next explored morphological differences in the DRN, regardless of condition, to see if this could explain observed sex differences in serotonergic activity. Four males were excluded from immunohistochemical analysis as their representative DRN sections had significantly larger areas than those of the rest of the sample. An unpaired t-test on

average DRN areas (mm^2) (Figure 3A) and TPH densities (TPH/mm^2) (Figure 3B) revealed that males and females had similar DRN anatomies and comparable TPH densities. Regardless of condition, males and females had different serotonergic modalities (Figure 3C). Females had higher percent TPH+Fos compared to males (p = 0.0227), but males had larger variation in serotonergic activity compared to females (p = 0.0180). These results indicate that DRN morphology alone cannot explain observed sex differences in behavior or serotonergic activity.

4.0 DISCUSSION

Based on previous research, serotonergic modulation may account for observed sex differences in fear discrimination, a cognitive function relevant to both typical behavior and anxiety disorders such as PTSD. We replicated sex differences in a fear discrimination paradigm and found via immunohistochemical analysis that females had a higher *level* of serotonergic activity compared to males, whereas males had larger *variation* of serotonergic activity compared to females.

From an adaptive perspective, females' rapid and robust discrimination between danger and safety may help conserve resources by eliciting appropriate behavioral responses only when necessary. Evolutionarily, females would have needed to recognize threats and react quickly to protect themselves and their young, as well as take advantage of safe opportunities to gather more resources to ensure their own and others' survival. Given enough time, males also learned to discriminate between danger and safety, but notably generalized the cues in the beginning of the conditioning session, going on to display higher freezing overall and never attaining the same level of discrimination of females. Males' inability to discriminate as well as females need not be seen as a disadvantage *per se*, as this behavior may stem from an evolutionary pressure to be more vigilant to address potential threats, such as an intruder to the nest or a predator while foraging.

While several studies have suggested potential sex differences in serotonergic modulation of fear behaviors, these studies have mainly considered the BLA. Mitsushima et al. (2006) found that male rats that underwent inescapable restraint stress had higher levels of extracellular 5-HT in the BLA as compared to those in similarly-treated female rats. However, while Duchesne et al. (2009) reported that females had higher levels of amygdaloid 5-HT, they also reported that males had higher levels of 5-HIAA in this structure, with a larger 5-HIAA/5-HT ratio indicative of higher 5-HT metabolism due to stress. The notion that females exhibit better fear discrimination, yet are more prone to depression and anxiety disorders in the human population, might be explained by this difference in 5-HT metabolic rate, where the prolonged presence of trace amounts of 5-HT in the BLA of females facilitates fear discrimination but has detrimental long-term effects.

Even within the raphe nuclei, there are different populations of serotonergic neurons that may modulate fear behaviors that our TPH label could not identify. Mamounas and Molliver's (1988) application of the mood-altering neurotoxin *p*-chloroamphetamine identified DRN-derived serotonergic "type D" axons that are more susceptible to degradation as compared to serotonergic "type M" axons that arise from the median raphe nucleus. As "type D" axons are selectively influenced by mood-altering compounds, this subpopulation may play a more dominant role in modulating emotional state, but may be influenced by its serotonergic neighbors. Lowry (2002) then proposed a model for parallel serotonergic circuits that allow integration of the behavioral, autonomic, and neuroendocrine responses associated with conditioned fear in the BLA. Following their tracing experiments highlighting the DRN-BLA system, Abrams et al.

(2005) measured increased serotonergic activity in the DRN after systemic administration of anxiogenic drugs using a TPH+Fos double label.

Acute SSRI treatment (Burghardt et al., 2004) and genetic downregulation of the 5-HT transporter and the degratory enzyme monoamine oxidase A (Garpenstrand et al., 2001) led to an increase in 5-HT levels and better fear discrimination, but a genetically induced decrease in 5-HT (Dai et al., 2008) was also able to produce this effect. To address these results, Homberg (2012) proposed a U-shaped relationship between 5-HT levels and the extent of fear conditioning, in which both too high and too low 5-HT levels increase associative learning and strengthen CS-US association. In our model, both high and low levels of 5-HT correlated with *poor* fear discrimination in males. To reconcile our model with that in Homberg's review, it is important to remember that our results were not influenced by pharmacological or genetic manipulations on the animals—serotonergic activity in the DRN-BLA system was left in its naturally occurring state. An overwhelming amount of 5-HT as a result of further experimental manipulations could upregulate the DRN-BLA system such that while the animal may successfully associate the CS with the US, it would also exhibit generally higher fear and an inability to effectively discriminate. Interestingly, males rats with the greatest CS+/CS- discrimination, in fact comparable to female behavior, had TPH+Fos levels that were very similar to females. This suggests that, in order to exhibit robust fear discrimination, there is an optimal level of 5-HT activity. Previous research demonstrated that manipulating serotonergic activity via a receptor antagonist (Foilb and Christianson, 2015) or a TPH inhibitor (Pettersson et al., 2016) improved fear discrimination in males; these mechanisms support our results of variable serotonergic activity and fear

discrimination in males and our conclusion that there exists an optimal 5-HT tone for males.

We found that neither a linear nor a nonlinear correlation between serotonergic activity and fear discrimination applied to females; instead, their collective activity was clustered in one region of the plot. This non-correlation implies that DRN activity does not usually play as large a role in the daily activities of females as it does in males, and that exposure to stress actually recruits DRN activity in a discrimination task in females. Activation of the DRN-BLA pathway in females may lead to a change in BLA plasticity mechanisms whereby they are more readily able to identify and distinguish between the CS+ and CS-.

Constant DRN activity in males, and therefore upregulation of the DRN-BLA pathway, supports the hypothesis that males evolved to be in a constant vigilant state. Assuming lower baseline DRN activity in females also corroborates the idea that the DRN is recruited only when the female must identify and react appropriately to either a threatening or harmless stimulus, and a narrow range of 5-HT activity in response to stress is also in line with females' assumed greater investment in conserving resources as compared to males.

Exposure to stress as a result of fear conditioning is also associated with the release of hormones such as norepinephrine and glucocorticoids, leading to the concurrent activation of β-adrenergic and glucocorticoid receptors within the BLA that mediates the establishment of long-term fear memory in the nucleus accumbens, hippocampus, entorhinal, and insular cortices (McGaugh, 2004). Subsequent ELISA testing would indicate whether males and females have different levels of stress

hormones as a result of fear discrimination. If males have an upregulated DRN-BLA system, then they may have a hormonal tone which is not enough to induce fear memory consolidation. On the other hand, the infrequent activation of the DRN-BLA system in females would lead to a sharp increase in stress hormone levels which may facilitate fear discrimination during the conditioning session. Previous studies (Rainnie, 1999; Wang and Aghajanian, 1977; Muller et al., 2007) in which DRN stimulation or exogenous 5-HT administration inhibited plasticity mechanisms in BLA did not consider the influence of stress hormones on the system.

These hypotheses cannot be proven until we have a better understanding of neural activity in fear behaviors. While the robust and cost-effective immunohistochemical labeling of Fos remains the most commonly used method for assessing neural activity, there are limitations that prevented us from acquiring as complete a picture of neural activation in the DRN as possible. First, while our TPH+Fos double label effectively identified the serotonergic neurons that were activated by fear discrimination, we do not know the phenotypes of the non-serotonergic neurons that were also activated. These neurons may be excitatory or inhibitory and may act on those serotonergic neurons, possibly drawing on other brain structures we did not consider in this study and thus adding another nuance to serotonergic modulation of fear discrimination. Secondly, Fos does not provide much information about the specific firing properties of the neurons that express it. Identifying Fos-labeled neurons with different-shaped action potentials and different firing patterns would provide more information about neural activity *in vivo* during fear discrimination. Lastly, Fos does not provide much information about the time, degree, and duration of activation, only that the neuron had been activated at some point

during the conditioning session. As a result, we do not know when or even if these neurons take part in the plasticity mechanisms that are implied in CS-US association, characteristic of learning and memory, and facilitatory in discriminating between the CS+ and CS-.

Knowledge of inherent sex differences in serotonergic activity and their possible implications in the ability to discriminate between what is safe from what is not augments our understanding of the higher prevalence of PTSD in women and addresses the urgent need to develop better treatments for the disorder. Estrogen has been found to increase TPH expression (Hiroi et al., 2006) and maintain serotonergic neurons in the DRN (Suzuki et al., 2013). Coupled with the fact that external social pressures may make women more susceptible to traumatic events as compared to men, extreme stress in women may upregulate the normally "quiet" DRN-BLA system to the point of maintaining abnormally high levels of 5-HT and stress hormones which lead to the onset of PTSD.

Despite their continued use, there is no consensus on the efficacy of SSRIs in treating anxiety and depressive-related symptoms of PTSD (Möller, 2009). Potential sex differences in 5-HT metabolism (Duchesne et al., 2009) would consequently lead to sex differences in SSRI mechanisms of action. If women with PTSD have higher levels of 5-HT and a lower rate of 5-HT metabolism due to stress as compared to men, then SSRIs, while proven effective for one biological sex, may actually be detrimental to the other. Based on our model, a more effective treatment for women with PTSD would be to develop pharmaceuticals that decrease serotonergic activity, whose efficacy can then be assessed using a fear discrimination paradigm.

A future direction for this sex differences study would be to apply the logic and technology of "engram" research (Denny et al., 2017; Kim and Cho, 2017) to induce activity-dependent labels in serotonergic cells involved in the DRN-BLA pathway to see if different neurons are involved in identifying and reacting to the CS+ versus the CS-, and, if they are different, to then quantify the amount of serotonin involved in modulating either circuit using high-performance liquid chromatography. Electrophysiological experiments would also help identify differences in the physiological properties of neurons due to sex and/or condition and confirm the extent to which synaptic plasticity—and therefore learning—is involved in fear discrimination. These methods can also be applied to animals subjected to the CS+ and "no shock" conditions to compare serotonergic modulation at another level of analysis.

5.0 REFERENCES

Abrams, J.K., Johnson, P.L., Hollis, J.H., Lowry, C.A. (2004). Anatomic and functional topography of the dorsal raphe nucleus. *Annals of the New York Academy of Sciences*. 1018: 46-57. doi: 10.1196/annals.1296.005.

Abrams, J.K, Johnson, P.L., Hay-Schmidt, A., Mikkelsen, J.D., Shekhar, A., Lowry, C.A. (2005). Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. *Neuroscience*. 133(4): 983-997. doi: 10.1016/j.neuroscience.2005.03.025.

Agnati, L.F., Zoli, M., Strömberg, I., Fuxe, K. (1995). Intercellular communication in the brain: wiring versus volume transmission. *Neuroscience*. 69(3): 711-726. doi: 0306-4522(95)00308-8.

Agnati, L.F., Guidolin, D., Guescini, M., Genedani, S., Fuxe, K. (2010). Understanding wiring and volume transmission. *Brain Research Reviews*. 64: 137-159. doi: 10.1016/j.brainresrev.2010.03.003.

Amat, J., Matus-Amat, P., Watkins, L.R., Maier, S.F. (1998). Escapable and inescapable stress differentially alter extracellular levels of 5-HT in the basolateral amygdala of the rat. *Brain Research*. 812(1-2): 113-120. doi: 10.1016/S0006-8993(98)00960-3.

Barnes, N.M., Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology*. 38: 1083-1152. doi: S0028-3908(99)00010-6.

Bocchio, M., McHugh, S.B., Bannerman, D.M., Sharp, T., Capogna, M. (2016). Serotonin, amygdala, and fear: assembling the puzzle. *Frontiers in Neural Circuits*. 10(24): 1-15. doi: 10.3389/fncir.2016.00024.

Boess, F.G., Martin, I.L. (1993). Molecular biology of 5-HT receptors. *Neuropharmacology*. 33(3-4): 275-317. doi: 0028-3908/94.

Breslau, N., Chilcoat, H.D., Kessler, R.C., Peterson, E.L., Lucia, V.C. (1999b). Vulnerability to assaultive violence: further specification of the sex difference in post-traumatic stress disorder. *Psychological Medicine*. 29(4): 813-821. doi: 10.1017/S0033291799008612.

Burghardt, N.S., Sullivan, G.M., McEwen, B.S., Gorman, J.M., LeDoux, J.E. (2004). The selective serotonin reuptake inhibitor citalopram increases fear after acute treatment but reduces fear with chronic treatment: a comparison with tianeptine. *Biological Psychiatry*. 55(12): 1171-1178. doi: 10.1016/j.biopsych.2004.02.029.

Burghardt, N.S., Bush, D.E.A., McEwen, B.S., LeDoux, J.E. (2007). Acute selective serotonin reuptake inhibitors increase conditioned fear expression: blockade with a 5-HT(2C) receptor antagonist. *Biological Psychiatry*. 62(10): 111-118. doi:10.1016/j.biopsych.2006.11.023.

Campbell, B.M.; Merchant, K.M. (2003). Serotonin 2C receptors within the basolateral amygdala induce acute fear-like responses in an open-field environment. *Brain Research*. 993(1-2): 1-9. doi: 10.1016/S0006-8993(03)03384-5.

Charnay, Y., Léger, L. (2010). Brain serotonergic circuitries. *Dialogues in Clinical Neuroscience*. 12(4): 471-487.

Christianson, J.P., Ragole, T., Amat, J., Greenwood B.N., Strong, P.V., Paul E.D., Fleshner, M., Watkins, L.R., Maier, S.F. (2010a). 5-hydroxytryptamine 2C receptors in the basolateral amygdala are involved in the expression of anxiety after uncontrollable traumatic stress. *Biological Psychiatry*. 67(4): 339-345. doi: 10.1016/j.biopsych.2009.09.011.

Commons, K.G., Connolley, K.R., Valentino, R.J. (2003). A neurochemically distinct dorsal raphe-limbic circuit with a potential role in affective disorders. *Neuropsychopharmacology*. 28: 206-215. doi: 10.1038/sj.npp.1200045.

Dai, J.X., Han, H.L., Tian, M. (2008). Enhanced contextual fear memory in central serotonin-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*. 105(33): 11981-11986. doi: 10.1073/pnas.0801329105.

Day, H.L.L., Reed, M.M. Stevenson, C.W. (2016). Sex differences in discriminating between cues predicting threat and safety. *Neurobiology of Learning and Memory*. 133: 196-203. doi: 10.1016/j.nlm.2016.07.014.

Denny, C.A., Lebois, E., Ramirez, S. (2017). From engrams to pathologies of the brain. *Frontiers in Neural Circuits*. 11(23): 1-20. doi: 10.3389/fncir.2017.00023.

Duchesne, A., Dufresne, M.M., Sullivan, R.M. (2009). Sex differences in corticolimbic dopamine and serotonin systems in the rat and the effect of postnatal handling. *Progress in Neuropsychopharmacology and Biological Psychiatry*. 33(2): 251-261. doi: 10.1016/j.pnpbp.2008.11.012.

Foilb, A.R., Christianson, J.P. (2015). Serotonin 2C receptor antagonist improves fear discrimination and subsequent safety recall. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. 65: 78-84. doi: 10.106/j.pnpbp.2015.08.017.

Foilb, A.R., Bals, J., Sarlitto, M.C., Christianson, J.P. (2017). Sex differences in fear discrimination do not manifest as differences in conditioned inhibition. *Learning & Memory*. 25: 1-6. doi: 10.1101/lm.045500.117.

Fuxe, K., Dahlström, A., Höistad, M., Marcellino, D., Jansson, A., Rivera, A., Diaz-Cabiale, Z., Jacobsen, K., Tinner-Staines, B., Hagman, B., Leo, G., Staines, W., Guidolin, D., Kehr, J., Genedani, S., Belluardo, N., Agnati, L.F. (2007). From the Golgi-Cajal mapping to the transmitter-based characterization of the neuronal networks leading to two modes of brain communication: wiring and volume transmission. *Brain Research Reviews*. 55: 17-54. doi: 10.1016/j.brainresrev.2007.02.009.

Garpenstrand, H., Annas, P., Ekblom, J., Oreland, L., Fredrikson, M. (2001). Human fear conditioning is related to dopaminergic and serotonergic biological markers. *Behavioral Neuroscience*. 115(2): 358-364. doi: 10.1037/0735-7044.115.2.358.

Giorgetti, M., Tecott, L. (2004). Contributions of 5-HT_{2C} receptors to multiple actions of central serotonin systems. *European Journal of Pharmacology*. 488: 1-9. doi: 10.1016/j/ejphar.2001.01.036.

Gutknecht, L., Popp, S., Waider, J., Sommerlandt, F.M.J., Göppner, C., Post, A., Reif, A., van den Hove, D., Strekalova, T., Schmitt, A., Colaço, M.B.N., Sommer, C., Palme, R., Lesch, K.P. (2015). Interaction of brain 5-HT synthesis deficiency, chronic stress and sex differentially impact emotional behavior in Tph2 knockout mice. *Psychopharmacology*. 232(14): 2429-2441. doi: 10.1007/s00213-015-3879-0.

Hale, M.W., Johnson, P.L., Westerman, A.M., Abrams, J.K., Shekhar, A., Lowry, C.A. (2010). Multiple anxiogenic drugs recruit a paravalbumin-containing subpopulaton of GABAergic interneurons in the basolateral amygdala. *Progressin Neuro-Psychopharmacology and Biological Psychiatry*. 34(7): 1285-1293. doi: 10.1016/j.pnpbp.2010.07.012.

Harris, J.A., Westbrook, R.F. (1999). The benzodiazepine midazolam does not impair Pavlovian fear conditioning but regulates when and where fear is expressed. *Journal of Experimental Psychology: Animal Behavior Processes*. 25(2): 236-246. doi: 10.1037/0097-7403.25.2.236.

Harris, J.A., Westbrook, R.F. (2001). Contextual control over the expression of fear in rats conditioned under a benzodiazepine. *Psychopharmacology*. 156:92-97. doi: 10.1007/s002130100757.

Hiroi, R., McDevitt, R.A., Neumaier, J.F. (2006). Estrogen selectively increases tryptophan hydroxylase-2 mRNA expression in distinct subregions of rat midbrain raphe nucleus: association between gene expression and anxiety behavior in the open field. *Biological Psychiatry*. 60: 288-295. doi: 10.1016/j.biopsych.2005.10.019.

Homberg, J. (2012). Serotonergic modulation of conditioned fear. *Scientifica*. 2012: 1-16. doi: 10.6064/2012/821549.

Howerton, A.R., Roland, A.V., Fluharty, J.M., Marshall, A., Chen, A., Daniels, D., Beck, S.G., Bale, T.L. (2014). Sex differences in corticotropin-releasing factor receptor-1 action within the dorsal raphe nucleus in stress responsivity. *Biological Psychiatry*. 75(11): 873-83. doi: 10.1016/j.biopsych.2013.10.013.

Jovanovic, T., Kazama, A., Bachevalier, J., Davis, M. (2012). Impaired safety signal learning may be a biomarker of PTSD. *Neuropharmacology*. 62(2): 695-704. doi: 10.1016/j.neuropharm.2011.02.023.

Kawahara, H., Yoshida, M., Yokoo, H., Nishi, M., Tanaka, M. (1993). Psychological stress increases serotonin release in the rat amygdala and prefrontal cortex assessed by in vivo microdialysis. *Neuroscience Letters*. 162(1-2): 81-84. doi: 10.1016/0304-3940(93)90565-3.

Kessler, R.C., Petukhova, M., Sampson, N.A., Zaslavsky, A.M., Wittchen, H.U. (2012). Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. *International Journals of Methods in Psychiatric Research*. 21(3): 169-184. doi: 10.1002/mpr.1359.

Kim, W.B., Cho, J.H. (2017). Encoding of discriminative fear memory by input-specific LTP in the amygdala. *Neuron*. 95: 1129-1146. doi: 10.1016/j.neuron.2017.08.004.

LeDoux, J. (2007). The amygdala. *Current Biology*. 17(20): 868-874. doi: 10.1016/j.cub.2007.08.005.

Lesch, K.P., Waider, J. (2012). Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. *Neuron*. 76(1): 175-191. doi: 10.1016/j.neuron.2012.09.013.

Li, Q., Luo, T., Jiang, X., Wang, J. (2012). Anxiolytic effects of 5-HT_{1A} receptors and anxiogenic effects of 5-HT_{2C} receptors in the amygdala of mice. *Neuropharmacology*. 62(1): 474-484. doi: 10.1016/j.neuropharm.2011.09.002.

Li, J., Liu, Q., Chen, Y., Liu, J., Shi, J., Liu, Y., Guo, J. (2016). Involvement of 5-HT_{1A} receptors in the anxiolytic-like effects of quercitrin and evidence of the involvement of the monoaminergic system. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2016/6530364.

Lisman, J., Schulman, H., Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature Reviews Neuroscience*. 3: 175-190. doi: 10.1038/nrn753.

Lowry, C.A. (2002). Functional subsets of serotonergic neurons: implications for control of the hypothalamic-pituitary-adrenal axis. *Journal of Neuroendocrinology*. 14(11): 911-923. doi: 10.1046/j.1365-2826.2002.00861.x

Lowry, C.A., Johnson, P.L., Hay-Schmidt, A., Mikkelsen, J., Shekhar, A. (2005). Modulation of anxiety circuits by serotonergic systems. *Stress*. 8(4): 233-246. doi: 10.1080/102553890500492787.

Maier, S.F., Watkins, L.R. (2005). Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neuroscience & Biobehavioral Reviews*. 29(4-5): 829-841. doi: 10.1016/j.neubiorev.2005.03.021.

Mamounas, L.A., Molliver, M.E. (1988). Evidence for dual serotonergic projections to neurocortex: axons from the dorsal and median raphe nuclei are differentially vulnerable to the neurotoxin *p*-chloroamphetamine (PCA). *Experimental Neurology*. 102(1): 23-36. doi: 10.1016/0014-4886(88)90075-1.

Manilow, R., Schulman, H., Tsien, R. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science*. 245(4290): 862-866. doi: 10.1126/science.2549638.

Marcondes, F.K., Bianchi, F.J., Tanno, A.P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Brazilian Journal of Biology*. 62(4A): 609-614. doi: 10.1590/S1519-69842002000400008.

Maren, S. (1999). Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends in Neuroscience*. 22(12): 561-567. doi: 10.1016/S0166-2236(99)01465-4.

McDonald, A.J., Augustine, J.R. (1993). Localization of GABA-like immunoreactivity in the monkey amygdala. *Neuroscience*. 52(2): 281-294. doi: 10.1016/0306-4522(93)90156-A.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience*. 27: 1-28. doi: 10.1146/annurev.neuro.27.070203.144157.

McKernan, M.G., Shinnick-Gallagher, P. (1997). Fear conditioning induces a lasting potentiation of synaptic currents *in vitro*. *Nature*. 390: 607-611. doi: 10.1038/37605.

Mitsushima, D., Yamada, K., Takase, K., Funabashi, T., Kimura, F. (2006). Sex differences in the basolateral amygdala: the extracellular levels of serotonin and dopamine, and their responses to restraint stress in rats. *European Journal of Neuroscience*. 24(11): 3245-3254. doi:10.111/j.1460-9568.2006.05214.x

Möller, H.J. (2009). Antidepressants: controversies about their efficacy in depression, their effect on suicidality and their place in a complex psychiatric treatment approach. *World Journal of Biological Psychiatry*. 10(3): 180-95. doi: 10.1080/15622970903101665.

Muller, J.F., Mascagni, F., McDonald, A.J. (2007). Serotonin-immunoreactive axon terminals innervate pyramidal cells and interneurons in the rat basolateral amygdala. *Journal of Comparative Neurology*. 505(3): 314-335. doi: 10.1002/cne.21486.

Ostroff, L.E., Cain, C.K., Bedont, J., Monfils, M.H., LeDoux, J.E. (2010). *Proceedings of the National Academy of Sciences of the United States of America*. 107(20): 9418-9423. doi: 10.1073/pnas.0913384107.

Pavlov, I.P. (1927). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. *Annals of Neurosciences*. 17(3): 136-141. doi: 10.5214/ans.0972-7531.1017309.

Paxinos, G., Watson, C. (2006). The Rat Brain in Stereotaxic Coordinates, 6th Edition. Academic Press. Amsterdam. The Netherlands.

Pettersson, R., Hagsäter, S.M., Eriksson, E. (2016). Serotonin depletion eliminates sex differences with respect to context-conditioned immobility in rat. *Psychopharmacology*. 233: 1513-1521. doi: 10.1007/s00213-016-4246-5.

Peyron, C., Petit, J.M., Rampon, C., Louvet, M., Luppi, P.H. (1997). Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience*. 82(2): 443-468. doi: 10.1016/S0306-4522(97)00268-6.

Rainnie, D.G. (1999). Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *Journal of Neurophysiology*. 82(1): 69-85. doi: 10.1153/jn.1999.82.1.69.

Reznikov, L.R., Reagan, L.P., Fadel, J.R. (2008). Activation of phenotypically distinct neuronal subpopulations in the anterior subdivision of the rat basolateral amygdala following acute and repeated stress. *Journal of Comparative Neurology*. 508(3): 458-472. doi:10.1016/j.pnpbp.2010.07.012.

Rogan, M.T., Stäubli, U.V., LeDoux, J.E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. *Nature*. 390: 604-607. doi: 10.1038/37601.

Rozeske, R.R., Evans, A.K., Frank, M.G., Watkins, L.R., Lowry, C.A., Maier, S.F. (2011). Uncontrollable, but not controllable, stress desensitizes 5-HT_{1A} receptors in the dorsal raphe nucleus. *Journal of Neuroscience*. 31(40): 14107-14115. doi: 10.1523/jneurosci.3095-11.2011.

Sanderson, J.L., Gorski, J.A., Dell'Acqua, M.L. (2016). NMDA receptor-dependent LTD requires transient synaptic incorporation of Ca^{2+}-permeable AMPARs mediated by

AKAP150-anchored PKA and calcineurin. *Neuron*. 89(5): 1000-1015. doi: 10.1016/j.neuron.2016.01.043.

Sanger, D.J., Joly, D. (1985). Anxiolytic drugs and the acquisition of conditioned fear in mice. *Psychopharmacology*. 85: 284-288. doi: 10.1007/BF00428188.

Sangha, S., Chadick, J.Z., Janak, P.H. (2013). Safety encoding in the basal amygdala. *Journal of Neuroscience*. 33(9): 3744-3751. doi: 10.1523/jneurosci.3302-12.2013.

Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K., Malinow, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science*. 284(5421): 1811-1816. doi: 10.1126/science.284.5421.1811.

Shin, R.M., Tsvetkov, E., Bolshakov, V.Y. (2006). Spatiotemporal asymmetry of associative synaptic plasticity in fear conditioning pathways. *Neuron*. 52(5): 883-896. doi: 10.1016/j.neuron.2006.10.010.

Smith, H.R., Porrino, L.J. (2008). The comparative distributions of the monoamine transporters in the rodent, monkey, and human amygdala. *Brain Structure and Function*. 213(1-2): 73-91. doi: 10.1007/s00429-008-0176-2.

Smith, Y., Paré, D. (1994). Intra-amygdaloid projections of the lateral nucleus in the cat: PHA-L anterograde labeling combined with postembedding GABA and glutamate immunocytochemistry. *Journal of Comparative Neurology*. 342(2): 232-48. doi: 10.1002/cne.903420207.

Stanton, P.K., Sarvey, J.M. (1984). Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *Journal of Neuroscience.* 4(12): 3080-3088. doi: 10.1523/jneurosci.04-12-03080.

Suzuki, H., Barros, R.P., Sugiyama, N., Krishnan, V., Yaden, B.C., Kim, H.J., Warner, M., Gustafsson, J.Å. (2013). Involvement of estrogen receptor β in maintenance of serotonergic neurons of the dorsal raphe. *Molecular Psychiatry*. 18(6): 674-680. doi: 10.1038/mp.2012.62.

Swanson, L.W., Petrovich, G.D. (1998). What is the amygdala? *Trends in Neurosciences*. 21(8): 323-331. doi: 10.1016/S0166-2236(98)01265-X.

Vicente, M.A., Zangrossi, H. (2012). Serotonin-2C receptors in the basolateral nucleus of the amygdala mediate the anxiogenic effect of acute imipramine and fluoxetine administration. *International Journal of Neuropsychopharmacology*. 15(3): 389-400. doi: 10.1017/S1461145711000873.

Vicente, M.A., Zangrossi, H. (2014). Involvement of 5-HT2C and 5-HT1A receptors of the basolateral nucleus of the amygdala in the anxiolytic effect of chronic antidepressant treatment. *Neuropharmacology*. 79: 127-135. doi: 10.1016/j.neuropharm.2013.11.007.

Walther, D.J., Bader, M. (2003). A unique central tryptophan hydroxylase isoform. *Biological Pharmacology*. 66: 1673-1680. doi:10.1016/S0006-2952(03)00556-2.

Wang, R.Y., Aghajanian, G.K. (1977). Inhibition of neurons in the amygdala by dorsal raphe stimulation: mediation through a direct serotonergic pathway. *Brain Research*. 120(1): 85-102. doi:10.1016/0006-8993(77)90499-1.

Zanoveli, J.M., Carvalho, M.C., Cunha, J.M., Brandão, M.L. (2009). Extracellular serotonin level in the basolateral nucleus of the amygdala and dorsal periaqueductal gray under unconditioned and conditioned fear states: an in vivo microdialysis study. *Brain Research*. 1294: 106-115. doi: 10.1016/j.brainres.2009.07.074.